Chapter 2

Dissection of Rodent Brain Regions: Guided Free-Hand Slicing and Dissection of Frozen Tissue

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Abstract

Previously we reported on a free-hand dissection guide for proteomics samples, where the major focus was on dissection of fresh brain tissue. Although the fresh brain provides clear advantages, such as being able to see structures in very great detail by differences in color and shade, it also has some disadvantages. This particularly becomes evident when processing multiple samples within a short time-window, e.g., after a physiological stimulus. In this chapter, we discuss some of the current methods to take tissue samples from frozen tissue, keeping in mind the requirements for subcellular isolation (e.g., synaptosomes) of proteomics samples. In addition, we provide a step-by-step protocol to improve standardization of tissue dissection in your lab.

Key words Guided free-hand dissection, Mouse, Rat, Brain, Frozen tissue

1 Introduction

Often, samples for various –omics techniques are harvested from frozen brain tissue. Specifically, smaller brain regions, e.g., amygdala, can be dissected or punched out in a relatively precise manner when using specific anatomical landmarks. As studying gene and protein expression after a physiological stimulus becomes more popular, the processing of these brain samples becomes a limiting factor. Hence, when timing is important and the total sample size (*n*-number, different genotypes or treatment groups) increases, it is easier to first freeze these brain samples and only later perform the dissections.

What makes sample preparation for proteomic special is that when one wants to obtain subcellular fractions, specific requirements come into place, in terms of maximal thickness of slices (300–400 μm). As such, the techniques described here, as well as the specific guided free-hand dissection method, focus on choices to be made on how to freeze the brain (*see* Subheading 1.1) and how to slice the brain (*see* Subheading 1.2). The various steps in

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the protocol (*see* Subheadings 2 and 3) provide detailed photographs alongside their corresponding anatomical pictures from the Allen brain atlas. It is important to note that although we detail our procedures for mouse tissue, a similar strategy can be implemented for any other species, provided that specific reference Atlases are available that are checked for strain, sex, and age.

1.1 What to Dissect and How to Freeze the Brain

As discussed before [1], dissecting regions of interest from frozen tissue has the advantage that more regions are accessible at the same time than with fresh tissue. Fresh dissection is less precise, and therefore should be focused on regions that are either hard to dissect in the frozen brain or that are relatively large, such as the dorsal striatum, thalamus, and the entire prefrontal cortex. In principle, with frozen dissection, various subregions can be harvested in parallel by precisely cutting out the region of interest from a brain slice, e.g., the prelimbic and infralimbic prefrontal cortex, and nucleus accumbens (ventral striatum).

Frozen tissue is especially suitable for tissue punching of small regions like the amygdala or ventral tegmental area (VTA). Note however, that specific anatomical features that are well distinguishable in the fresh brain might be harder to see in frozen sections (Fig. 1). As such, clear anatomical hallmarks must be used to ensure precise dissection. In this protocol we will discuss several steps to decrease this possible source of variation.

Prior to material collection, it is wise to consider the type of freezing protocol to be utilized and the orientation in which the brain is frozen. With regards to freezing protocols, a routine technique involves snap-freezing tissue into relatively inert cryogenic liquids, such as liquid nitrogen (−196 °C), or in 2-methylbutane

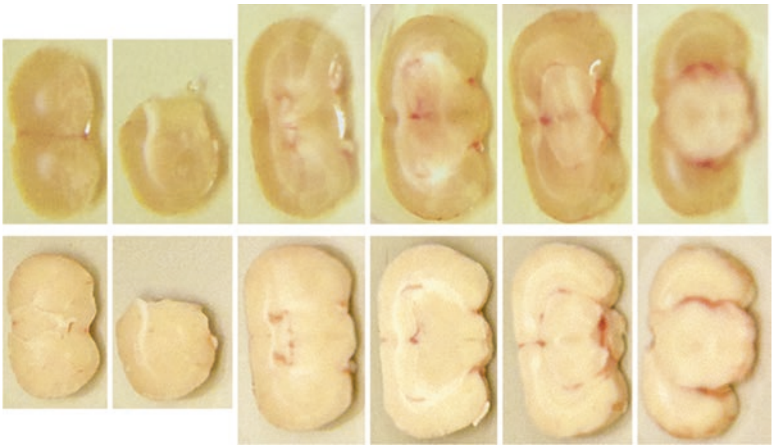

Fig. 1 Difference in ability to observe anatomical landmarks in frozen and "fresh" mouse brain sections. The same frozen sections (lower) were thawed (upper) in order to view the distinction in color and anatomical landmarks. The thawed version was covered in Aquatex in order to prevent drying during photography

(formerly called isopentane) that is precooled (−78 °C) in dry-ice sludge. The advantage of snap-freezing protocols is that it keeps degradation of proteins or RNA to a minimum, while preserving the structure well. However, there are a few disadvantages attached to these methods in relation to subsequent slicing for tissue dissection; the entire brain is frozen in the form it was just prior to freezing, hence special care should be taken to maintain anatomical integrity. Another disadvantage is that due to the unequal speed of freezing, friction within the brain might lead to tissue cracks. This typically occurs with larger samples like the rat brain, but may also happen with the mouse brain. When solely used for dissection this does not have to be a problem, although specific anatomical landmarks might be harder to see. Furthermore, both cryogenic liquids are highly hazardous (Box 1); liquid nitrogen due to its low temperature and liquid-to-gas expansion ratio and 2-methylbutane due to its extremely high toxicity and burden on the environment. Lastly, when used for free-hand slicing, as we discuss here, the natural bulged forms of the brain will make it hard to keep it in a steady position during slicing on a flat surface/carrier. Therefore, we will discuss a fast-freezing option using dry-ice (Box 1) only. Note, however, that slower freezing, than that used with cryogenic liquids, could result in the formation of ice-crystals within the brain that may severely damage its (ultra)structure. This is of importance when the brain specimen is used for both proteomics sampling and for immunological staining of tissue sections. For details see Cunningham & Doran 2012 (https://www.leicabiosystems.com/pathologyleaders/freezing-biological-samples/).

Box 1: Cryogenic Liquids and Solids for Brain Freezing

Liquid nitrogen:

Has a high liquid-to-gas expansion ratio at room temperature. Therefore, breaking a bucket of liquid nitrogen in a small room can yield a high pressure. Moreover, it can then be an asphyxiant. Watch out for cold-burns when spilling droplets of liquid on unprotected skin.

2-Methylbutane/Methylbutane/Isopentane:

Is extremely volatile and flammable (GHS category H224) at room temperature. Best to store cooled, especially when using it as cryogenic compound. Toxicity for aspiration (H304, H336) and the burden on the aquatic environment (H411) form major disadvantages of its use, and urge for alternatives.

Dry ice (solid CO2):

Sublimates into CO_2 gas, leaving no residual. Watch out for cold-burns when touching the pellets.

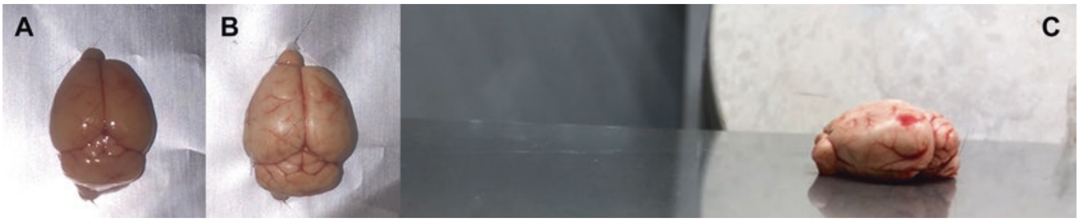

Fig. 2 Fast-freezing the mouse brain on dry ice. The brain is put in the desired position while on normal ice (**a**) and frozen on dry ice (**b**) while being on the same carrier. The aluminum foil prevents the brain from sticking to the carrier and can be used to immediately wrap the brain for storage purposes. This procedure keeps the frozen brain in a steady position for subsequent (guided) free-hand slicing (**c**)

The dry-ice freezing option uses a limited set of materials (*see* below) and typically takes ~1–2 min longer than the snap-freezing method with size of tissue influencing freezing time. For this procedure, the brain is taken from the skull and put on a piece of aluminum foil on a metal carrier in the desired orientation/position, while being cooled on normal ice. Subsequently the foil and carrier are placed on dry ice, allowing the brain to freeze (Fig. 2a, b), where the speed of freezing can be adjusted by placing dry ice parts or pellets near the brain. As such, the brain will freeze and be maintained in a steady position (Fig. 2c) for subsequent free-hand slicing. It is, however, important to keep in mind that the way of positioning the brain (ventral part up, or dorsal part up) influences the regions to be dissected. For example, when the region of interest is ventrally located, it is wiser to freeze the brain with the ventral surface facing up because (1) contact with the metal carrier deforms normal anatomy and (2) with freehand slicing standardized vertical cuts are often hard to maintain and so linear and volumetric variations in the plane of slicing tend to be the smallest closer to the surface from where slices are made.

A last important step is storage of the collected brain samples. Mostly, bulky samples like entire brains are stored at −80 °C, where it is essential to protect the sample from freeze-drying, a process that occurs at low temperatures over time. Therefore, every brain should be wrapped in a piece of aluminum foil. Either write the animal code on the foil and/or add a pencil- or laser-printed paper slip to it for long-term storage and retrieval. Store all brain specimens together in a carton/plastic box (−80 °C) and add a pencil- or laser-printed experiment description.

1.2 How to Slice the Brain?

The most important choice to make is the method used to slice the brain. In principle, there are four options detailed in Box 2. Each option has advantages and disadvantages, but due to its relative simplicity, low costs but good possibility to standardize, we will discuss option 4; using a self-constructed device for guided free-hand slicing.

Box 2: Slicing Options for Frozen Brain

Cryostat:

Check your departments' cryostat for the option to make thick (>200 μm) sections. Older machines can be 'preloaded' for example by turning the wheel 3× without cutting and reversing it and making the actual cut at the 4th turn (set at 50 μm). Slice at −12 to −10 °C. Check whether this option is suitable for your samples with a few test brains first.

Advantage: Very precise slicing. Ideal for relatively thin slices (<100 μm). Works better for rat than for mouse brains due to size.

Disadvantage: Time-consuming, for thicker slices of a mouse brain (>100–200 μm), it is essential to embed the brain in OCT compound's like Tissue-Tek®, to prevent curling and crumbling of brain slices. Thin slices are harder to process for subcellular fractionation in proteomics.

For the options below, one needs a cooling device that is ideally set at −12 to −10 °C. In principle this is possible in a cryostat. Note however that modern cryostats have specific temperature controls for the tissue specimen stage, whereas the chamber itself is at −20 °C, which is too cold. Also keep in mind that you will need a metal non-iron plate for this, in order to not damage the cryostat table. Always check whether this option is suitable for your samples with a few test brains first. It is not possible to perform simultaneous multiple slicing as the low temperature will deform the entire brain by the pressure put onto it. Lowering the temperature (−15 °C) results in more difficulty to slice and hence more pressure needed. The protocol described in this chapter in more detail uses the guided free-hand slicing and dissection.

Brain slicer matrix:

Best to combine with a cryogenic liquid freezing protocol, due to the curvature of the matrix.

Advantage: Relatively precise slicing, thinner than the free-hand options. Different options are available for different sized brains. Relatively standardized slicing.

Disadvantage: Expensive device. Difficult to hold the brain in the same position while cutting.

Freehand slicing:

Combine with dry-ice freezing protocol.

Advantage: Cheapest and easiest option. Ideal when dissecting large tissue chunks.

Disadvantage: Not precise. Large variation from sample-to-sample and between people.

Guided free-hand slicing:

Combine with dry-ice freezing protocol. Self-make your own slice-guide.

(continued)

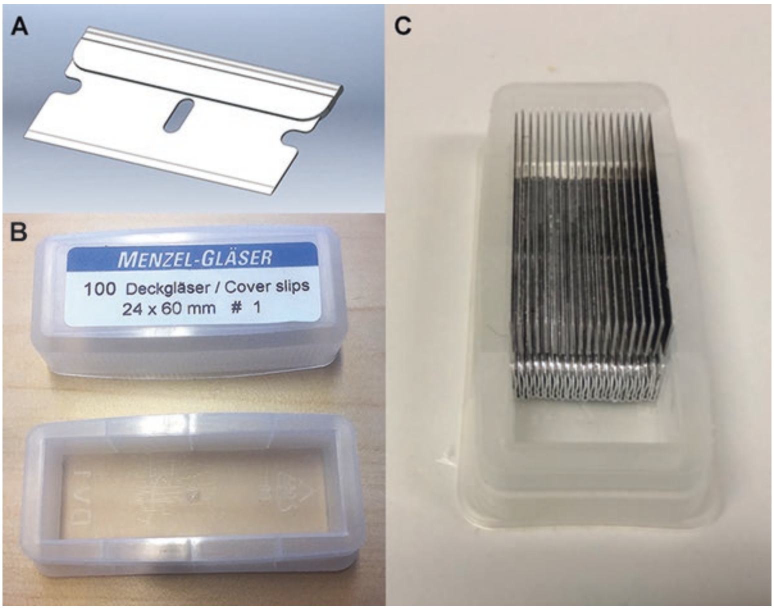

Fig. 3 Free-hand slicing guide. (**a**, **b**) Standard single-edge razor blade (e.g., Fisherbrand 12–640; **a**) was pasted into a microscopy cover slip box (**b**). (**c**) This specific guide contains 19 blades with an inter-blade distance of ~1 mm

Advantage: Cheap and easy construction for cutting guide. Flexible in size. Easy to use. Improved precision over free-hand slicing and hence provides medium-high standardization of sample collection.

Disadvantage: Cutting distance is determined by the width of the single edge blade (Fig. 4). Yet creative minds might see a way around this limitation.

This device (Fig. 3) will be used to make precuts in the frozen brain to guide subsequent free-hand slicing (Fig. 4). For standardization purposes, it is important to achieve a constant inter-blade distance. In this case, this was achieved by pasting 19 standard single-edge razor blades into a cover slip box (Fig. 3), creating a distance of ~1 mm, where the number of blades depends on the size of the lid you put the blades in. In our case, the rims on the outside of the box enable easy handling of the device.

It is important to adjust the type of slicing and the standardization to your needs. In Fig. 4, we have indicated to have one precut at a fixed position, i.e., at the most posterior contact side of left and right cerebrum (Fig. 4c) or at the border of the most anterior side of the hypothalamus at the side of the optic chiasm (Fig. 4d).

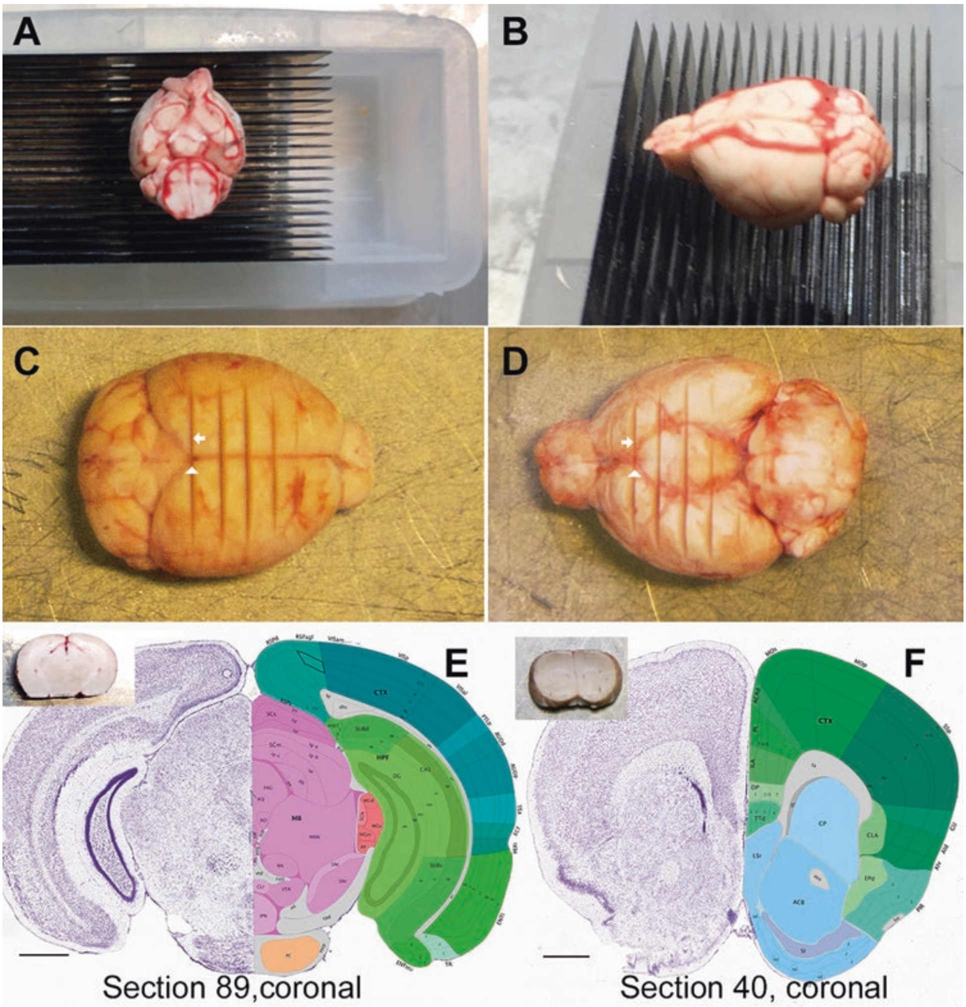

Fig. 4 Free-hand slicing guide in action. (**a**–**d**) Brains for dorsal dissection (**a**, **b**) or ventral dissection (**b**, **d**) were precut (**c**, **d**, respectively) at a fixed anatomical position (arrowhead). Note the visible contact site of freezing and subsequent deformation at the ventral and dorsal site in panels **a** and **b**, respectively. Subsequently, using a separate blade, free-hand coronal slices were generated based on the precuts, while keeping the brain on a metal non-iron plate in a cryostat (−10 °C). (**e**, **f**) The corresponding Allen Brain reference atlas picture of the slice (arrows indicate direction of view) indicated in panels **c**, **d** is shown, with the actual slice as inset

2 Materials

The products used are listed below. Comparable products from other suppliers should also be effective. Equipment visible on the photographical and schematic representation of the dissections (Figs. 3–6, 9) is indicated by ∗.

2.1 Brain Removal

See description given in Spijker 2011 [1].

2.2 Brain Freezing

1. Favorably 2–3 metal iron-free plates [1]. Alternatively, take a solid plastic item and wrap aluminum foil firmly around it. Always keep a separate piece of foil to put the brain on.
2. Aluminum foil in pieces of ~6 × 6 cm (mouse) or 10 × 10 cm (rat).
3. Forceps as indicated previously [1].
4. Dry ice pellets in a polystyrene box with lid.

2.3 Brain Dissection

1. Favorably 1 metal iron-free plate [1]. Alternatively, take a solid plastic item, and wrap aluminum foil firmly around it. Due to brain slicing this latter material is not preferred.
2. Cotton gloves (e.g., Cara cotton gloves (medium, Walmart #551140545)) to be worn under normal Nitrile or Latex gloves to reduce heat transfer and to stay comfortable while dissecting in a cryostat.
3. Razor blades (e.g., Fisherbrand 12–640).
4. Scalpel handle (FST # 10003–12 Scalpel Handle #3—12 cm) with scalpel (FST # 10011–00), as illustrated in Spijker 2011,

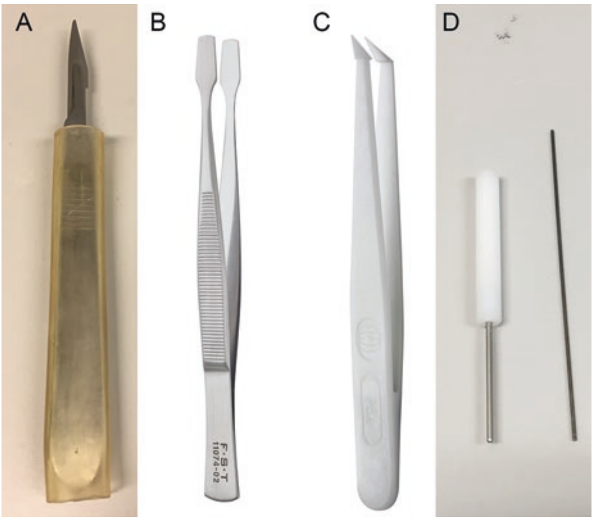

Fig. 5 Specific dissection tools. (**a**) Scalpel handle (FST # 10003–12 Scalpel Handle #3—12 cm) with scalpel (FST # 10011–00), where the handle is covered with clear PVC tubing to reduce heat transfer while dissecting. (**b**, **c**) Forceps to keep the brain in a steady position while free-hand slicing; straight cover glass forceps (FST11074–02, **b**). To reduce heat transfer while dissecting the brain, consider the plastic (Acetyl) version (FST11700–02; **c**). Although it has a smaller surface, this 45° angled version has a pointed tip that eases picking up the dissected brain areas. (**d**) Tissue puncher (FST # 18036–19) and metal expeller

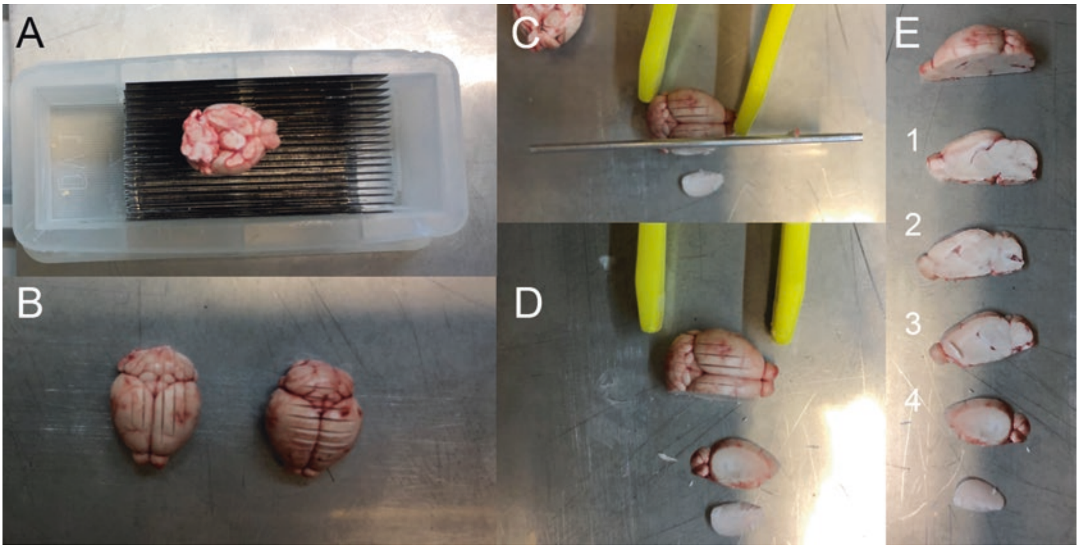

Fig. 6 Generation of a digitally archived sagittal series. (**a**, **b**) The brain (frozen with ventral side down) is precut with the slice-guide (**a**, **b**). (**c**, **d**) Subsequently, while holding the brain with a plastic forceps, sagittal sections are made using a razor blade (**c**, **d**). (**e**) The consecutive sections are numbered (1–4) and are detailed upon in Fig. 7

Fig. 2c [1]. A plastic handle (FST # 10000–11) of the disposable scalpels reduces heat transfer to the brain sample during dissection, and hence might be the preferred choice.

5. Optional: Use clear PVC (Vinyl) tubing to cover the stainless-steel handle of FST # 10003–12 and hence have a cheap and endurable solution to reduce heat transfer (Fig. 5a).
6. Straight forceps with larger surface area to firmly hold the brain without damaging it. The stainless steel version FST11074–02 (straight cover glass forceps; Fig. 5b) would suffice, but very suitable plastic versions (e.g., FST11700–02; Figs. 5c and Fig. 6) are available as well.
7. Tissue puncher (FST # 18036–19 for 1 mm ø), if applicable for the type and size of tissue (e.g., amygdala, VTA). Note that it creates a bit of damage (~0.2–0.6 mm) to the surrounding tissue,
8. Create the guided dissection tool with the following (*see* Subheading 1.2 and Fig. 3):
    - Microscopic coverslip box. Menzel-Gläser, 24 × 60, #1 (e.g., VWR 631–0973; Fig. 3b) ∗. Alternative: use the 25 × 50, #1 (e.g., VWR 631–0854).
    - Standard razor blades (Fisherbrand 12–640).
    - Glue.

9. Atlases to compare your slices with; e.g., mouse brain digital ones from Allen Brain Atlas (http://mouse.brain-map.org/static/atlas) or from Matt Gaidica based on Paxinos and Franklin 2001 [2] (http://labs.gaidi.ca/mouse-brain-atlas), as well as the rat version based on Paxinos and Watson 2006 [3] (http://labs.gaidi.ca/rat-brain-atlas). The Allen Brain atlas pictures have the advantage that specific subregions can be highlighted and are easy to find. The Paxinos picture indicates both the distance relative to Bregma and the Interaural point, as well as the medial-lateral distance (in mm). Both mouse atlases are based on a P56 mouse brain, hence variation in the exact coordinates may occur when using older animals.

3 Methods

3.1 Brain Removal

See description given in Spijker 2011 [1].

3.2 Brain Freezing

1. Prepare (incl. Marking for specimen) aluminum foil pieces to wrap the frozen brain, as well as preparing paper slip (*see* Subheading 1.1) to mark the brain specimen (genotype, treatment, experiment, animal number).
2. Place the aluminum foil on the metal iron-free plate that is on ice.
3. Transfer the brain after removal from the skull onto the aluminum foil. Place with the dorsal side up when the area of interest is on the dorsal side.
4. Transfer the entire plate onto the dry ice (pellets) to freeze slowly. One can increase freezing speed by adding pellets onto the plate in close vicinity (but not touching) the brain.
5. Depending on the species (mouse ~2–4 min; rat ~3–6 min) fold the aluminum foil (with paper slip) to show the experimental marks (e.g., animal number) visible to ease later retrieval of a specific specimen.

3.3 Brain Dissection

It is advisable to practice the use of dissection several times. When sufficient reproducibility is achieved for brains of a specific strain, genotype, sex, and age, it is advisable to generate a standard brain series (Figs. 6 and 7) where the anterior and posterior sides of each slice is archived digitally and compared with available brain atlases. For such a series, one could make the frozen and the thawed version, as each provides clear and valuable anatomical hallmarks (*see* Fig. 7).

1. Transfer the aluminum foil-wrapped brains on dry ice to the cryostat.
2. Put the temperature of the cryostat at −10 °C (or the preferred slicing temperature).

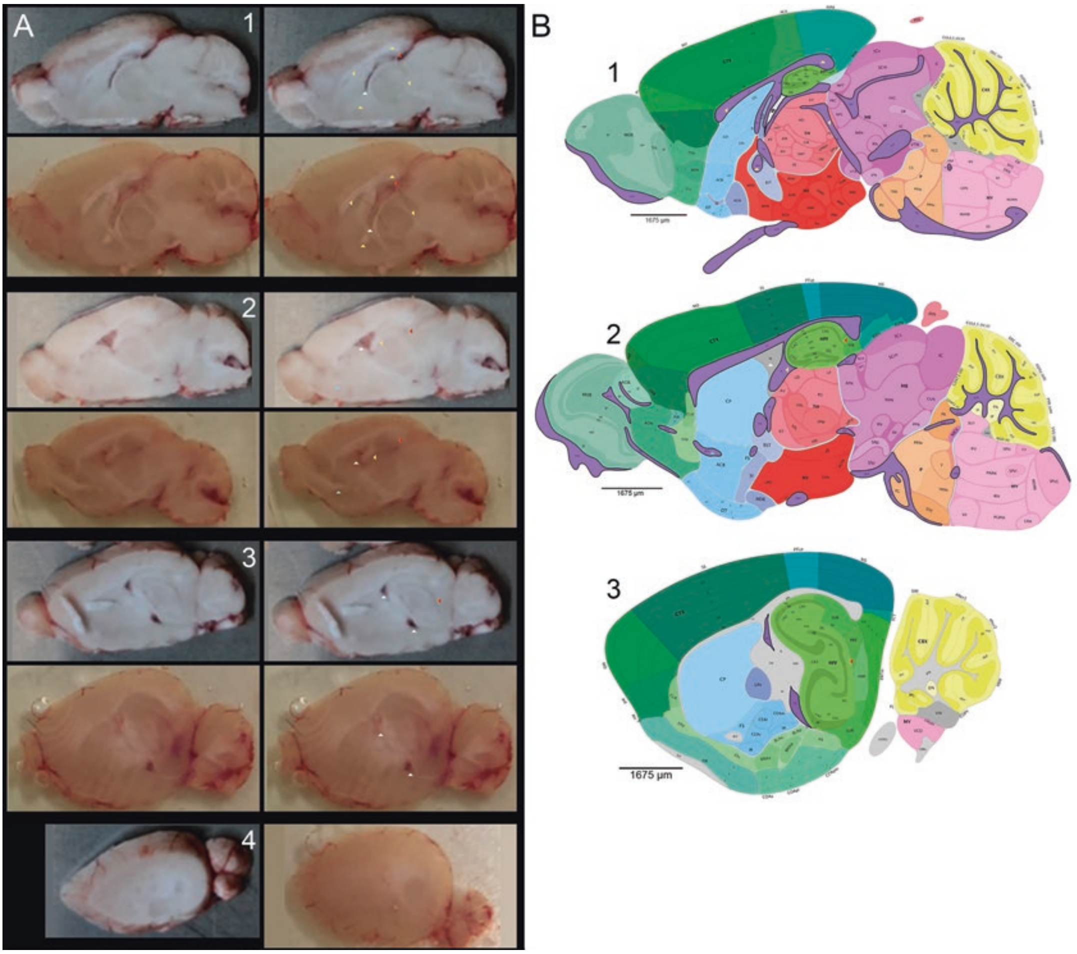

Fig. 7 Digitally archived sagittal series. (**a**) Frozen sections labeled 1–4, see Fig. 6, are depicted, as well as the thawed version covered with Aquatex. All pictures are duplicated to show specific anatomical landmarks on the right side. **a1**: Third ventricle (white arrowhead), various white matter tracts (yellow arrowheads), and hippocampus (red arrowhead with yellow line) are indicated. Note that specific features are also visible on section 19 of the Allen Brain atlas (not shown here). **a2**: Lateral ventricle (white arrowhead), the fimbria (yellow arrowhead), the anterior commissure (turquois arrowhead), and hippocampus (red arrowhead with yellow line). **a3**: Dorsal and ventral part of the lateral ventricle (white arrowheads) between the white matter of, e.g., fimbria and internal capsule visible (not indicated), and hippocampus (red arrowhead with yellow line). Note that specific features (e.g., the shape of the hippocampus) are better visible on section 5 of the Allen Brain atlas (not shown here). (**b**) The corresponding Allen Brain reference atlas picture of slices 1–3 (section 18, 14, and 6, respectively) are shown, as slice 4 falls outside the range depicted in the Allen Brain Atlas. In **b1** and **b2** the white matter tracts/fibers are highlighted in purple, and in **b1** the third ventricle (V3) is highlighted in white. In **b3** the lateral ventricle is highlighted in purple. Note. Arrowheads as depicted in (**a**) are visible at the corresponding spot in (**b**)

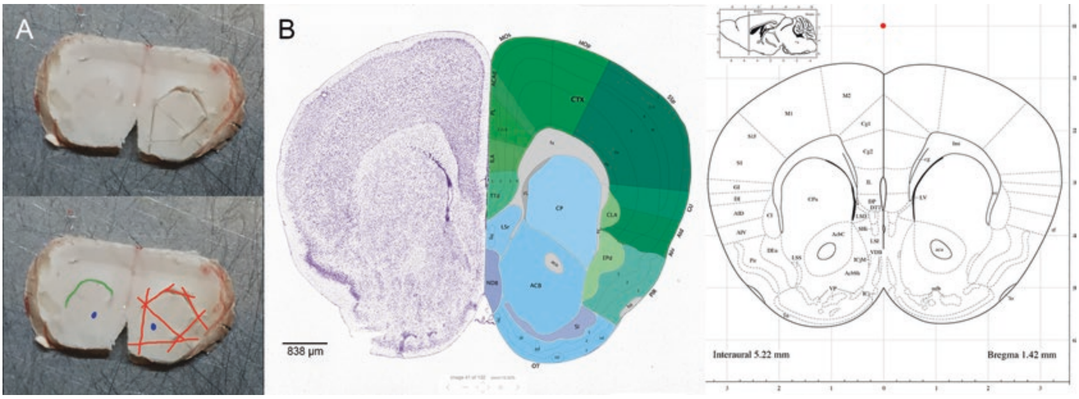

Fig. 8 Dissection of dorsal and ventral striatum. (**a**) Brain slice similar to the one shown in Fig. 4d, f. The lower picture shows the same slice but now with marker lines indicating the border of the dorsal striatum and fmi (green), the anterior commissure (aca; blue), and the incisions made to obtain the dorsal (caudate putamen, CP(u)) and ventral (nucleus accumbens (ACB/Acb), core and shell) part of the striatum. Incisions were made using a scalpel and knife (FST # 10011–00, see Fig. 5a). Note that depending on the thickness of the slice and the anterior–posterior differences in size of the brain area, these incisions can be made on either side, where cutting in an angle could help to adjust for these differences. (**b**) Digital atlas pictures (*left, Allen Brain section 40; lower, Paxinos figure 19 by Matt Gaidica*) corresponding to the anterior side of the slice

3. Place a metal non-iron plate on the cryostat table. After 2 min place the brains on the plate, and let them acclimatize for 2–3 h.
4. Place all dissection tools in the cryostat ~5 min before the start.
5. Prepare Eppendorf vials to hold the dissected brain areas. Label all tubes well, both on the top and the side. Note that it might be handy to dissect more areas from the same slice, even if the tissue is not used immediately. Especially for large experiments, it is ethically preferable to use these additional brain areas for a pilot study at a later moment in time, rather than to restart a new experiment with new animals.
6. Unwrap the first brain and use the dissection guide to make incisions on the dorsal or ventral part (*see* Fig. 4).
7. Dissect (Fig. 8) or punch out the region of interest (Fig. 9).
8. Gather the tissue of the region of interest in Eppendorf tubes, store at −80 °C until further processing.
9. After the preferred brain areas have been taken out, put back the remainder of the brain (if applicable) in the aluminum foil and store at −80 °C for later use.

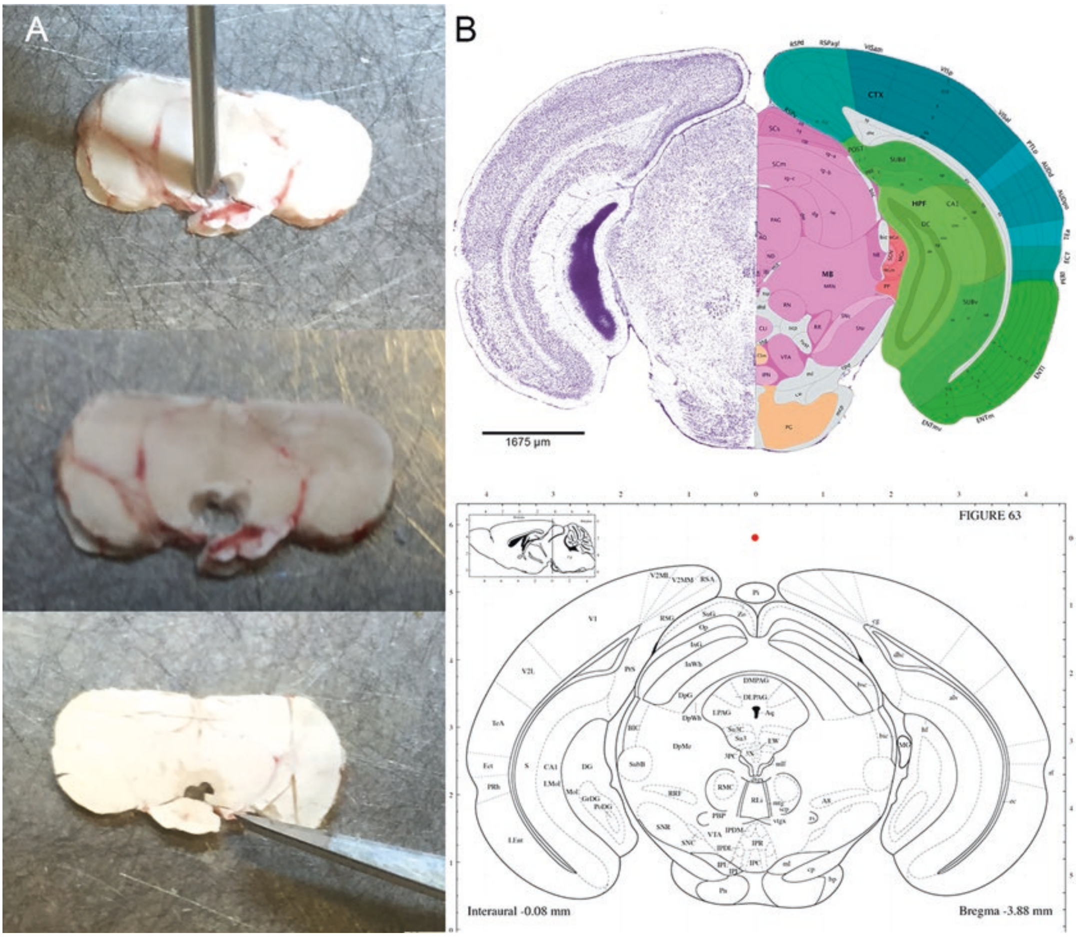

Fig. 9 Punching out VTA-enriched mid-brain. (**a**) The posterior side from the last coronal section was used to punch out VTA-enriched mid-brain (*upper two pictures*) into the anterior direction using a 1 mm ø tissue puncher (FST # 18036–19). On the anterior side (*bottom picture*), the punch is visible as well. Note that a start was made in dissecting the ventral subiculum on the right side. (**b**) Digital atlas pictures (*upper, Allen Brain section 90; lower, Paxinos figure 63 by Matt Gaidica*) corresponding to the posterior side of the slice

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